

NOVEL PEPTIDE-FORMING ENZYME, MICROBE PRODUCING THE  
ENZYME AND METHOD FOR PRODUCING PEPTIDE USING THEM

CROSS-REFERENCE TO RELATED APPLICATIONS

5         The present application claims priority to U.S. provisional application 60/491,554, filed on August 1, 2003. The present application is also related to Japanese patent application JP 2002-218956, filed on July 26, 2002. The entire contents of the US 60/491,554 and JP 2002-218956 is hereby incorporated by reference in  
10         their entirety.

BACKGROUND OF THE INVENTION

1) Field of the Invention

15         The present invention relates to a novel enzyme that can form a peptide easily, inexpensively and at high yield without going through a complex synthesis method. More particularly, the present invention relates to a novel enzyme that catalyzes a peptide-forming reaction from a carboxy component and an amine component, to a microbe that produces the enzyme, and a method for producing a dipeptide using the  
20         enzyme or microbe.

2) Description of the Related Art

Peptides are used in the fields of pharmaceuticals, foods and various other fields. For example, since L-alanyl-L-glutamine has  
25         higher stability and water-solubility than L-glutamine, it is widely used

as a component of fluid infusion and serum-free media.

Chemical synthesis methods, which have been known as methods for producing peptides, are not always easy. Known examples of such methods include a method that uses

- 5 N-benzyloxycarbonylalanine (hereinafter, "Z-alanine") and protected L-glutamine (see Bull. Chem. Soc. Jpn., 34, 739 (1961), Bull. Chem. Soc. Jpn., 35, 1966 (1962)), a method that uses Z-alanine and protected L-glutamic acid- $\gamma$ -methyl ester (see Bull. Chem. Soc. Jpn., 37, 200 (1964)), a method that uses Z-alanine ester and unprotected
- 10 glutamic acid (see Japanese Patent Application Laid-open Publication No. H1-96194), a method that involves synthesis of an N-(2-substituted)-propionyl glutamine derivative as an intermediate from a 2-substituted-propionyl halide as a raw material (see Patent Application Laid-open Publication No. H6-234715).

- 15 However, since all of these methods require the introduction and elimination of protecting groups or the use of an optically active intermediate, they are not considered to be adequately satisfactory in terms of their industrial advantages.

- 20 On the other hand, widely known examples of typical peptide production methods using enzymes consist of a condensation reaction that uses an N-protected and C-unprotected carboxy component and an N-unprotected, C-protected amine component (hereinafter, "Reaction 1"), and a substitution reaction that uses an N-protected, C-protected carboxy component and an N-unprotected, C-protected amine
- 25 component (hereinafter, "Reaction 2"). An example of Reaction 1 is a

method for producing Z-aspartylphenylalanine methyl ester from Z-aspartic acid and phenylalanine methyl ester (see Japanese Patent Application Laid-open Publication No. S53-92729), while an example of Reaction 2 is a method for producing acetylphenylalanylleucine amide

5 from acetylphenylalanine ethyl ester and leucine amide (see Biochemical J., 163, 531 (1977)). There have been reported very few research examples of methods that use an N-unprotected, C-protected carboxy component. An example of a substitution reaction that uses an N-unprotected, C-protected carboxy component and an N-unprotected,

10 C-protected amine component (hereinafter, "Reaction 3") is described in International Patent Publication WO 90/01555. For example, a method for producing arginylleucine amide from arginine ethyl ester and leucine amide may be mentioned of. Examples of substitution reactions that use an N-unprotected, C-protected carboxy component and an

15 N-unprotected, C-unprotected amine component (hereinafter, "Reaction 4") are described in European Patent Publications EP 278787A1 and EP 359399B1. For example, a method for producing tyrosylalanine from tyrosine ethyl ester and alanine may be mentioned of.

20 **SUMMARY OF THE INVENTION**

The most inexpensive production method among the aforementioned methods of Reactions 1 to 4 naturally falls within the class of Reaction 4, which involves the fewest protecting groups.

However, the example of Reaction 4 of the prior art (European Patent Publication EP 278787A1) had the following major problems:

(1) extremely slow rate of peptide formation,  
(2) low yield of peptide formation,  
(3) the peptides that can be produced are limited to those that contain amino acids with comparatively high hydrophobicity,  
5 (4) the amount of enzyme added is extremely large, and  
(5) comparatively expensive carboxypeptidase preparations derived from molds, yeasts or plants are required. In the Reaction 4, there is no method known whatsoever that uses an enzyme derived from bacteria or yeasts other than the genus *Saccharomyces*, and there is no  
10 known method for producing alanylglutamine and other peptides that are highly hydrophilic. In consideration of this background, there is a need to develop an industrially inexpensive method for producing these peptides.

It is an object of the present invention is to provide a novel  
15 enzyme that can form a peptide easily, inexpensively and at high yield without going through a complex synthesis method. More particularly, it is an object of the present invention to provide a novel enzyme that catalyzes a peptide-forming reaction from a carboxy component and an amine component, a microbe that produces the enzyme, and a method  
20 for inexpensively producing peptide using the enzyme or microbe.

The inventors of the present invention have found a novel enzyme that efficiently forms peptide from newly discovered bacteria belonging to the genus *Empedobacter* and so forth, and have completed the present invention.

25 The present invention is as described below:

[1] An enzyme derived from a microbe belonging to a genus selected from the genus *Empedobacter* and the genus *Sphingobacterium*, having the ability to form a peptide from a carboxy component and an amine component.

5 [2] An enzyme having the ability to form a peptide from a carboxy component and an amine component and the ability to form L-alanyl-L-glutamine at a formation rate of 0.03 mM/min or more in a dipeptide-forming reaction under the conditions (i) to (iv):

(i) the carboxy component is L-alanine methyl ester hydrochloride in  
10 an amount of 100 mM;

(ii) the amine component is L-glutamine in an amount of 200 mM;

(iii) the pH is 9.0; and,

(iv) the amount of homogeneously purified enzyme added is less than 0.61 mg/ml as protein.

15 [3] The enzyme according to [1] or [2], wherein the carboxy component as a substrate includes both the amino acid ester and the amino acid amide.

[4] The enzyme according to any one of [1] to [3], wherein any of an amino acid, a C-protected amino acid and an amine can be used as a  
20 substrate for the amine component.

[5] The enzyme according to any one of [1] to [4], wherein the enzyme has the ability to form a peptide within a pH range of 6.5 to 10.5.

[6] The enzyme according to any one of [1] to [5], wherein the  
25 enzyme has the ability to form a peptide within a temperature range of 0

to 60°C.

[7] The enzyme according to any one of [1] to [6], wherein the enzyme is not inhibited by a serine enzyme inhibitor, phenylmethylsulfonyl fluoride, but is inhibited by

5    p-nitrophenyl-p'-guanidinobenzoate.

[8] The enzyme according to any one of [1] to [7], wherein the enzyme has a molecular weight as determined by SDS-gel electrophoresis of about 75 kilodaltons, and a molecular weight as determined by gel filtration chromatography of about 150 kilodaltons.

10    [9] A microbe that produces an enzyme according to any one of [1] to [8].

[10] The microbe according to [9], wherein, the microbe is *Empedobacter brevis* strain FERM BP-8113 (Depository institution: the independent administrative corporation, National Institute for Advanced

15    Industrial Science and Technology, International Patent Organism Depository, Address of deposited institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date: July 8, 2002) or *Sphingobacterium sp.* strain FERM BP-8124 (Depository institution: the independent administrative

20    corporation, National Institute for Advanced Industrial Science and Technology, International Patent Organism Depository, Address of depository institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date: July 22, 2002).

[11] A method for producing a dipeptide, comprising forming a

25    dipeptide from a carboxy component and an amine component using an

enzyme according to any one of [1] to [8] or a substance containing the enzyme.

The other objects, features and advantages of the present invention are specifically set forth in or will become apparent from the 5 following detailed descriptions of the invention when read in conjunction with the accompanying drawings.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a diagram showing an optimum pH of the enzyme of the 10 present invention;

Fig. 2 is a diagram showing an optimum temperature of the enzyme of the present invention; and

Fig. 3 is a diagram showing time-dependent the time course of L-alanyl-L-glutamine production from L-alanine methyl ester and 15 L-glutamine.

#### DETAILED DESCRIPTION

Modes for carrying out the present invention are explained in detail in the order of

- 20 (1) Microbe Producing the Enzyme of the Present Invention,
- (2) Microbe Culturing,
- (3) Enzyme Purification,
- (4) Enzyme Properties, and
- (5) Dipeptide Synthesis Method.

(1) Microbe Producing the Enzyme of the Present Invention

The enzyme of the present invention may be any enzyme that has the ability to form a peptide from a carboxy component and an amine component, and there is no particular restriction on organisms 5 that produce such an enzyme. In the present specification, the carboxy component refers to the component that provides a carbonyl site (CO) in the peptide bond (-CONH-), while the amine component refers to the component that provides the amino site (NH) in the peptide bond. In addition, in the present specification, the term "peptide" used 10 alone refers to a polymer having at least one peptide bonds unless otherwise indicated specifically. In addition, the term "dipeptide" in the present specification refers to a peptide having one peptide bond.

Examples of microbes that produce the enzyme of the present invention include bacteria belonging to the genus *Empedobacter* and so 15 forth, specific examples of which include *Empedobacter brevis* strain ATCC 14234 (strain FERM P-18545), and *Sphingobacterium* sp. strain FERM BP-8124. *Empedobacter brevis* strain ATCC 14234 (strain FERM P-18545, strain FERM BP-8113) and *Sphingobacterium* sp. strain FERM BP-8124 are microbes that were screened by the inventors of the 20 present invention as a result of searching for microbes that produce a peptide from a carboxy component and an amine component at high yield. Microbes having similar bacteriological properties to those of *Empedobacter brevis* strain ATCC 14234 (strain FERM P-18545) or *Sphingobacterium* sp. strain FERM BP-8124 are also microbes that 25 produce the enzyme of the present invention.

*Empedobacter brevis* strain ATCC 14234 (strain FERM P-18545) was deposited at the International Patent Organism Depository of the National Institute for Advanced Industrial Science and Technology (Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan) on 5 October 1, 2001 and assigned the deposit number of FERM P-18545. Control of this organism was subsequently transferred to deposition under the provisions of the Budapest Treaty at the International Patent Organism Depository of the National Institute for Advanced Industrial Science and Technology on July 8, 2002 and was assigned the deposit 10 number of FERM BP-8113 (indication of microbe: *Empedobacter brevis* strain AJ 13933).

*Sphingobacterium sp.* strain AJ 110003 was deposited at the International Patent Organism Depository of the National Institute for Advanced Industrial Science and Technology on July 22, 2002, and was 15 assigned the deposit number of FERM BP-8124. It should be noted that strain AJ 110003 was identified to be the aforementioned *Sphingobacterium sp.* by the identification experimentation described below. Strain FERM BP-8124 (Depository institution: the independent administrative corporation, National Institute for Advanced Industrial 20 Science and Technology, International Patent Organism Depository, Address of depository institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit date: July 22, 2002) is a Gram-negative rod (0.7 to 0.8 × 1.5 to 2.0 micrometers (μm)) that does not form spores and is not motile. Its colonies are 25 round with a completely smooth border, contain low protrusions and

have a glossy, light yellow color. The organism grows at 30°C and is catalase positive, oxidase positive and negative for the OF test (glucose), and was identified as a bacterium belonging to the genus *Sphingobacterium* based on these properties. Moreover, from the 5 properties of being negative for acid nitrate reduction, negative for indole production, negative for production from glucose, arginine dihydrolase negative, urease positive, esculin hydrolysis positive, gelatin hydrolysis negative,  $\beta$ -galactosidase positive, glucose assimilation positive, L-arabinose assimilation negative, D-mannose 10 assimilation positive, D-mannitol assimilation negative, N-acetyl-D-glucosamine assimilation positive, maltose assimilation positive, potassium gluconate assimilation negative, n-capric acid negative, adipic acid assimilation negative, dl-malic acid assimilation negative, sodium citrate assimilation negative, phenyl acetate 15 assimilation negative and cytochrome oxidase positive, the microbe was determined to have properties that are similar to those of *Sphingobacterium multivorum* or *Sphingobacterium spiritivorum*. Moreover, as a result of analyzing homology of the base sequence of 20 16S rRNA gene, although the highest degree of homology was exhibited with *Sphingobacterium multivorum* (98.8%), there was no strain with which it matched completely. This bacterial strain was therefore identified as *Sphingobacterium* sp.

The enzyme of the present invention can be obtained by isolating and purifying from the cells of the above-mentioned 25 *Empedobacter brevis* or *Sphingobacterium* sp. In addition, the enzyme

of the present invention as well as microbes that produce the enzyme can also be obtained by genetic engineering techniques based on the isolated enzyme. Namely, the enzyme and microbe of the present invention can be produced by isolating a DNA that encodes the enzyme 5 of the present invention based on the isolated and purified enzyme followed by expressing the DNA by introducing the DNA into a suitable host. In addition, an enzyme having the ability to form a peptide from a carboxy component and an amine component can also be obtained from other microbes by producing a probe based on a polynucleotide 10 and so forth that encodes the enzyme of the present invention obtained from *Empedobacter brevis*. Various gene recombination techniques are described in Molecular Cloning, 2nd edition, Cold Spring Harbor Press (1989) and other publications.

#### (2) Microbe Culturing

15 In order to obtain cultured cells of microbes having the enzyme used in the present invention, it suffices that the microbes be cultured and grown in a suitable medium. There is no particular restriction on the medium used for this purpose so far as it allows the microbes to grow. This medium may be an ordinary medium containing ordinary 20 carbon sources, nitrogen sources, phosphorus sources, sulfur sources, inorganic ions, and organic nutrient sources as necessary.

For example, any carbon source may be used so far as the microbes can utilize it. Specific examples of the carbon source that can be used include sugars such as glucose, fructose, maltose and amylose, 25 alcohols such as sorbitol, ethanol and glycerol, organic acids such as

fumaric acid, citric acid, acetic acid and propionic acid and their salts, hydrocarbons such as paraffin as well as mixtures thereof.

Examples of nitrogen sources that can be used include ammonium salts of inorganic acids such as ammonium sulfate and

5 ammonium chloride, ammonium salts of organic acids such as ammonium fumarate and ammonium citrate, nitrates such as sodium nitrate and potassium nitrate, organic nitrogen compounds such as peptones, yeast extract, meat extract and corn steep liquor as well as mixtures thereof.

10 In addition, ordinary nutrient sources used in media, such as inorganic salts, trace metal salts and vitamins, can also be suitably mixed and used.

There is no particular restriction on culturing conditions, and culturing can be carried out, for example, for about 12 to about 48

15 hours while properly controlling the pH and temperature to a pH range of 5 to 8 and a temperature range of 15 to 40°C, respectively, under aerobic conditions.

### (3) Enzyme Purification

A method for isolating and purifying a peptide-forming enzyme

20 from *Empedobacter brevis* is explained as an example of purifying the enzyme of the present invention. First, a microbial cell extract is prepared from the microbial cells of *Empedobacter brevis*, for example, the strain FERM BP-8113 (Depository institution: the independent administrative corporation, National Institute for Advanced Industrial

25 Science and Technology, International Patent Organism Depository,

Address of deposited institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date: July 8, 2002) and the like by disrupting the microbial cells using a physical method such as ultrasonic crushing or an enzymatic method

5 using a cell wall-dissolving enzyme and removing the insoluble fraction by centrifugal separation and so forth.

A peptide-forming enzyme can then be purified from the cell extract obtained in the above manner by combining ordinary protein purification methods such as anion exchange chromatography, cation

10 exchange chromatography or gel filtration chromatography.

An example of a carrier for use in anion exchange chromatography is Q-Sepharose HP (manufactured by Amersham). The enzyme is recovered in the non-adsorbed fraction under conditions of pH 8.5 when the cell extract containing the enzyme is allowed to

15 pass through a column packed with the carrier.

An example of a carrier for use in cation exchange chromatography is MonoS HR (manufactured by Amersham). After adsorbing the enzyme onto the carrier (in the column) by allowing the cell extract containing the enzyme to pass through a column packed

20 with the carrier and then washing the column, the enzyme is eluted with a buffer solution having a high salt concentration. At that time, the salt concentration may be sequentially increased or gradiently increased. For example, in the case of using MonoS HR, the enzyme adsorbed onto the carrier is eluted at an NaCl concentration of about 0.2 to about

25 0.5 M.

The enzyme purified in the manner described above can then be further homogeneously purified by gel filtration chromatography and so forth. An example of the carrier for use in gel filtration chromatography is Sephadex 200pg (manufactured by Amersham).

5        The fraction that contains the present enzyme in the aforementioned purification procedure can be confirmed by assaying the peptide production activity of each fraction according to the method described later.

#### (4) Properties of Enzyme of the Present Invention

10        While the enzyme of the present invention is an enzyme that has the ability to form a peptide from a carboxy component and an amine component, a preferable mode of the enzyme of the present invention will be explained hereinbelow from the standpoint of its properties.

15        An enzyme having the abilities described below, for which the dipeptide formation rate is used as an indicator, is one preferable mode of the enzyme of the present invention. Namely, a preferable mode of the enzyme of the present invention is an enzyme that has the ability to form a peptide from a carboxy component and an amine component, 20 and the ability to exhibit a formation rate of L-alanyl-L-glutamine of preferably 0.03 mM/min or more, more preferably 0.3 mM/min or more, and particularly preferably 1.0 mM/min or more in the dipeptide-forming reaction under the conditions of (i) to (iv) below. The conditions of the dipeptide-forming reaction are as follows:

25        (i) The carboxy component is L-alanine methyl ester hydrochloride

(100 millimolar (mM));

- (ii) The amine component is L-glutamine (200 mM);
- (iii) The pH is 9.0; and,
- (iv) The amount of homogeneously enzyme added is less than 0.61

5 mg/ml as protein amount.

The aforementioned amount of enzyme added indicates a final amount of added enzyme that is added to the reaction system, and addition of the enzyme of 0.01 mg/ml or more, and preferably 0.02 mg/ml or more, as protein amount is desirable. The term "protein 10 amount" refers to the value indicated with the Coomassie brilliant blue colorimetric method using protein assay CBB solution (manufactured by Nakarai) and bovine serum albumin for the standard substance.

The aforementioned formation rate far exceeds the conventional formation rate for peptide formation using an enzyme, and the enzyme 15 of the present invention has the ability to catalyze peptide production at an extremely high rate.

As a specific example of a procedure for assaying enzyme activity, enzyme activity can be assayed by allowing the enzyme to react in a borate buffer solution containing an amino acid ester and an 20 amine as substrates followed by quantifying the resulting peptide. As a more specific example, the enzyme is allowed to react for several minutes at 25°C using a 100 mM borate buffer solution (pH 9.0) containing 100 mM L-alanine methyl ester and 200 mM L-glutamine.

The enzyme activity unit used in the present invention is defined 25 such that 1 unit (U) is the amount of enzyme that forms 1 micromole of

peptide in 1 minute under the condition of reacting at 25°C using 100 mM borate buffer solution (pH 9.0) containing 100 mM L-alanine methyl ester and 200 mM L-glutamine.

In addition, a preferable mode of the enzyme of the present invention is an enzyme having the property by which both an amino acid ester and amino acid amide can be used as a substrate for the carboxy component. The words "both an amino acid ester and an amino acid amide can be used as a substrate" mean that at least one type of amino acid ester and at least one type of amino acid amide can be used as a substrate. In addition, one preferable mode of the enzyme of the present invention is an enzyme that has the property by which any of an amino acid, a C-protected amino acid and an amine can be used as a substrate for the amine component. The words "an amino acid, a C-protected amino acid, and an amine can be used as a substrate" mean that at least one type of amino acid, at least one type of C-protected amino acid and at least one type of amine can be used as a substrate. As a result of having a wide range of substrate specificity with respect to the carboxy component or the amino component, the enzyme of the present invention is preferable in the sense that a wide range of raw materials can be selected, which in turn is favorable in terms of cost and production equipment in the case of industrial production.

Specific examples of carboxy components include L-amino acid esters, D-amino acid esters, L-amino acid amides and D-amino acid amides. In addition, amino acid esters include not only amino acid

esters corresponding to naturally-occurring amino acids, but also amino acid esters corresponding to non-naturally-occurring amino acids or their derivatives. Further, examples of amino acid esters include  $\alpha$ -amino acid esters as well as  $\beta$ -,  $\gamma$ -, and  $\omega$ -amino acid esters and the like, which have different amino group bonding sites. Typical examples of amino acid esters include methyl esters, ethyl esters, n-propyl esters, iso-propyl esters, n-butyl esters, iso-butyl esters and tert-butyl esters of amino acids, etc.

Specific examples of the amine components include L-amino acids, C-protected L-amino acids, D-amino acids, C-protected D-amino acids and amines. In addition, examples of the amines include not only naturally-occurring amines, but also non-naturally-occurring amines or their derivatives. In addition, examples of the amino acids include not only naturally-occurring amino acids, but also non-naturally-occurring amino acids or their derivatives. These include  $\alpha$ -amino acids as well as  $\beta$ -,  $\gamma$ - and  $\omega$ -amino acids and the like, which have different amino group bonding sites.

In addition, in a different aspect, one preferable mode of the enzyme of the present invention is an enzyme in which the pH range over which the peptide-forming reaction can be catalyzed is 6.5 to 10.5. The ability of the enzyme of the present invention to catalyze this reaction over a wide pH range is preferable in that it allows flexible accommodation of industrial production that could be subject to the occurrence of various restrictions. However, in the actual production of peptide, it is preferable to use the enzyme by further adjusting to an

optimum pH corresponding to the acquired enzyme so as to maximize the catalytic performance of the enzyme.

Moreover, another different aspect of a preferable mode of the enzyme of the present invention is an enzyme for which the

5 temperature range over which the enzyme is capable of catalyzing the peptide-forming reaction is within the range of 0 to 60°C. Since the enzyme of the present invention is able to catalyze the reaction over a wide temperature range, it is preferable in that it allows flexible accommodation of industrial production that could be subject to the

10 occurrence of various restrictions. However, in the actual production of peptides, it is preferable to use the enzyme by further adjusting to an optimum temperature corresponding to the acquired enzyme so as to maximize the catalytic performance of the enzyme.

#### (5) Dipeptide Synthesis Method

15 The method for producing a dipeptide according to the present invention comprises synthesizing a dipeptide by allowing an enzyme having the ability to form a peptide from a carboxy component and an amine component or a substance that contains that enzyme to act on the carboxy component and the amine component.

20 For the method for allowing the enzyme used in the present invention or enzyme-containing substance to act on the carboxy component and the amine component, it suffices that the enzyme or enzyme-containing substance, carboxy component and amine component be mixed. More specifically, a method may be used in

25 which an enzyme or enzyme-containing substance is added to a

solution containing the carboxy component and the amine component and allowed to react, or in the case of using a microbe that produces the enzyme, a method may be used in which the microbe that produces the enzyme is cultured, the enzyme present in the microbe or microbial culture fluid is produced and accumulated, and the carboxy component and amine component are then added to the culture fluid. The formed dipeptide can then be collected by established methods and purified as necessary.

The term "enzyme-containing substance" refers to that which contains the enzyme, and examples of specific forms thereof include a culture of microbes that produce the enzyme, microbial cells isolated from the culture, and a treated microbial cell product. A culture of microbes refers to that which is obtained by culturing microbes, and more specifically, to a mixture of microbial cells, medium used for culturing the microbes, and substances produced by the cultured microbes and the like. In addition, the microbial cells may be washed and used in the form of washed microbial cells. Further, a treated microbial cell product includes the crushed, lysed or freeze-dried microbial cells, and also includes a crude enzyme recovered by processing microbial cells and so forth as well as a purified enzyme obtained by purification of the crude enzyme. A partially purified enzyme obtained by various types of purification methods may be used for the purified enzyme, or immobilized enzyme may be used that has been immobilized by covalent bonding, adsorption or entrapment methods. In addition, since some microbes are partially lysed during

culturing depending on the microbes used, the culture supernatant may also be used as the enzyme-containing substance in such cases.

In addition, not only wild strains but also genetic recombinant strains may be used for the microbes that contain the enzyme. The 5 microbes are not limited to intact cells, but rather acetone-treated microbial cells, freeze-dried microbial cells or other treated microbial cells may also be used. Immobilized microbial cells immobilized by covalent bonding, adsorption, entrapment or other methods, as well as treated immobilized microbial cells, may also be used.

10 The term "homogeneously purified enzyme" as used herein refers to an enzyme that shows a homogeneous band in an electrophoresis experiment of a purified protein containing an enzyme protein and has enzyme activity of that enzyme.

It should be noted that in the case of using cultures, cultured 15 microbial cells or a treated microbial cell product, there are many cases in which an enzyme exists that does not participate in production of peptides but decomposes the formed peptides, and in such cases, it is rather preferable to add a metal protease inhibitor like ethylenediaminetetraacetic acid (EDTA) depending on the cases. The 20 addition amount is within the range of 0.1 mM to 300 mM, and preferably 1 mM to 100 mM.

The amount of enzyme or enzyme-containing substance used 25 should be an amount at which the target effect is demonstrated (hereinafter, "effective amount"). Although this effective amount can be easily determined through simple, preliminary experimentation by a

person with ordinary skill in the art, in the case of using an enzyme, for example, the use amount thereof is about 0.01 to 100 units (U), while in the case of using washed microbial cells, the use amount thereof is about 1 to 500 g/L.

5 Any carboxy component may be used so far as it is capable of producing a peptide by condensation with the other substrate in the form of the amine component. Examples of the carboxy components include L-amino acid esters, D-amino acid esters, L-amino acid amides and D-amino acid amides. Examples of the amino acid esters include  
10 not only amino acid esters corresponding to naturally-occurring amino acids, but also amino acid esters corresponding to non-naturally-occurring amino acids or their derivatives. In addition, examples of the amino acid esters include  $\alpha$ -amino acid esters as well as  $\beta$ -,  $\gamma$ - and  $\omega$ -amino acid esters and the like, which having different  
15 amino group bonding sites. Typical examples of the amino acid esters include methyl esters, ethyl esters, n-propyl esters, iso-propyl esters, n-butyl esters, iso-butyl esters and tert-butyl esters of amino acids.

Any amine component may be used so far as it is capable of producing peptide by condensation with the other substrate in the form  
20 of the carboxy component. Examples of the amine components include L-amino acids, C-protected L-amino acids, D-amino acids, C-protected D-amino acids and amines. In addition, examples of the amines include not only naturally-occurring amines, but also non-naturally-occurring amines or their derivatives. Examples of the  
25 amino acids include not only naturally-occurring amino acids, but also

non-naturally-occurring amino acids or their derivatives. These include  $\alpha$ -amino acids as well as  $\beta$ -,  $\gamma$ - and  $\omega$ -amino acids and the like, which have different amino group bonding sites.

Although the concentrations of the carboxy component and 5 amine component serving as starting materials are 1 mM to 10 M, and preferably 0.05 mole (M) to 2 M, respectively, there are cases in which it is preferable to add the amine component in an amount equal to or greater than that of the carboxy component. In addition, in the case of substrates that inhibit the reaction at high concentrations, these can be 10 adjusted to a concentration that does not result in inhibition and successively added during the reaction.

The reaction temperature that allows production of a peptide is 0 to 60°C, and preferably 5 to 40°C. The reaction pH that allows production of a peptide is 6.5 to 10.5, and preferably 7.0 to 10.0.

15

### Examples

Although the following provides a more detailed explanation of the present invention through its examples, the present invention is not limited to these examples. In addition to confirmation by ninhydrin 20 coloring of thin-film chromatograms (qualitative), quantitative determinations were made by the following high-performance liquid chromatography in order to assay products. Column: Inertsil ODS-2 (manufactured by GL Science, Inc.), eluate: aqueous phosphate solution containing 5.0 mM sodium 1-octanesulfonate (pH 2.1) : 25 methanol = 100 : 15 to 50, flow rate: 1.0 mL/min, detection: 210

nanometers (nm).

Example 1 Microbe Culturing (*Empedobacter brevis* Strain FERM BP-8113)

5 A 50 mL medium (pH 6.2) containing 5 grams (g) of glucose, 5 g of ammonium sulfate, 1 g of monopotassium phosphate, 3 g of dipotassium phosphate, 0.5 g of magnesium sulfate, 10 g of yeast extract and 10 g of peptone in 1 liter (L) was transferred to a 500 mL Sakaguchi flask and sterilized at 115°C for 15 minutes. This medium  
10 was then inoculated with one ml of culture broth of *Empedobacter brevis* strain FERM BP-8113 (Depositary institution: the independent administrative corporation, National Institute for Advanced Industrial Science and Technology, International Patent Organism Depositary, Address of depositary institution: Chuo Dai-6, 1-1 Higashi 1-Chome, 15 Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date: July 8, 2002) that had been cultured at 30°C for 16 hours in the same medium, followed by shake culturing at 30°C for 16 hours and 120 strokes/min.

20 Example 2 Production of Peptide Using Microbial Cells

Microbial cells were collected by centrifuging (10,000 rounds per minute (rpm), 15 minutes) the culture broth obtained in Example 1 followed by suspending to a concentration of 100 g/L in 100 mM borate buffer (pH 9.0) containing 10 mM EDTA. After respectively adding 1 25 mL of this suspension to 1 mL of 100 mM borate buffer (pH 9.0)

containing 10 mM EDTA, 200 mM of the following carboxy component and 400 mM of the following amino acids to bring to a final volume of 2 mL, the reaction was carried out at 18°C for 2 hours. The peptides that were formed as a result of this reaction are shown in Table 1.

5

Table 1

Carboxy component	Amine component	Produced peptide (mM)		Carboxy component	Amine component	Produced peptide (mM)	
L-Ala-OMe	L-Leu	L-Ala-L-Leu	38.2	Gly-OMe	L-His	L-Gly-L-His	22.1
	L-Met	L-Ala-L-Met	68.3	L-Ser-OMe	L-Ser	L-Ser-L-Ser	29.0
	L-Phe	L-Ala-L-Phe	62.4	L-Val-OMe	L-Met	L-Val-L-Met	10.5
	L-Ser	L-Ala-L-Ser	51.3	L-Met-OMe	L-Phe	L-Met-L-Phe	28.5
	L-His	L-Ala-L-His	52.1	L-Thr-OMe	L-Leu	L-Thr-L-Leu	23.0
	L-Arg	L-Ala-L-Arg	72.1	L-Ile-OMe	L-Met	L-Ile-L-Met	8.3
	L-Gln	L-Ala-L-Gln	68.0				

(Hydrochloride salts were used for all the carboxy components.)

### Example 3 Enzyme Purification

The procedure after centrifugal separation was carried out either

10 on ice or at 4°C. *Empedobacter brevis* strain FERM BP-8113  
 (Depository institution: the independent administrative corporation, National Institute for Advanced Industrial Science and Technology, International Patent Organism Depository, Address of depository institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken,, 15 Japan, International deposit transfer date: July 8, 2002) was cultured in

the same manner in as Example 1, and the microbial cells were collected by centrifugal separation (10,000 rpm, 15 minutes). After washing 16 g of microbial cells with 50 mM Tris-HCl buffer (pH 8.0), they were suspended in 40 milliliters (ml or mL) of the same buffer and 5 subjected to ultrasonic crushing treatment for 45 minutes at 195 watts. This ultrasonic crushing liquid was then centrifuged (10,000 rpm, 30 minutes) to remove the crushed cell fragments and obtain an ultrasonic crushing liquid supernatant. This ultrasonic crushing liquid supernatant was dialyzed overnight against 50 mM Tris-HCl buffer (pH 10 8.0) followed by removal of the insoluble fraction by ultracentrifugation (50,000 rpm, 30 minutes) to obtain a soluble fraction in the form of the supernatant liquid. The resulting soluble fraction was applied to a Q-Sepharose HP column (manufactured by Amersham) pre-equilibrated 15 with Tris-HCl buffer (pH 8.0), and the active fraction was collected from the non-adsorbed fraction. This active fraction was dialyzed overnight against 50 mM acetate buffer (pH 4.5) followed by removal of the insoluble fraction by centrifugal separation (10,000 rpm, 30 minutes) to 20 obtain a dialyzed fraction in the form of the supernatant liquid. This dialyzed fraction was then applied to a Mono S column (manufactured by Amersham) pre-equilibrated with 50 mM acetate buffer (pH 4.5) to elute enzyme at a linear concentration gradient of the same buffer containing 0 to 1 M NaCl. The fraction that had the lowest level of 25 contaminating protein among the active fractions was applied to a Superdex 200pg column (manufactured by Amersham) pre-equilibrated with 50 mM acetate buffer (pH 4.5) containing 1 M NaCl, and gel

filtration was performed by allowing the same buffer (pH 4.5) containing 1 M NaCl to flow through the column to obtain an active fraction solution. As a result of performing these procedures, the peptide-forming enzyme used in the present invention was confirmed to 5 have been uniformly purified based on the experimental results of electrophoresis. The enzyme recovery rate in the aforementioned purification process was 12.2% and the degree of purification was 707 times.

#### 10 Example 4 Measurement of Enzyme Molecular Weight

##### SDS-Gel Electrophoresis

A 0.3 microgram ( $\mu$ g) of the purified enzyme fraction obtained by the method of Example 3 was applied to polyacrylamide electrophoresis. 0.3% (w/v) Tris, 1.44% (w/v) glycine and 0.1% (w/v) sodium 15 laurylsulfate were used for the electrophoresis buffer solution, a gel having a concentration gradient of a gel concentration of 10 to 20% (Multigel 10 to 20, manufactured by Daiichi Pure Chemicals) was used for the polyacrylamide gel, and Pharmacia molecular weight markers were used for the molecular weight markers. Following completion of 20 electrophoresis, the gel was stained with Coomassie brilliant blue R-250, and a uniform band was detected at the location of a molecular weight of about 75 kilodalton (kDa).

##### Gel filtration

The purified enzyme fraction obtained by the method of Example 25 3 was applied to a Superdex 200pg column (manufactured by

Amersham) pre-equilibrated with 50 mM acetate buffer (pH 4.5) containing 1 M NaCl, and gel filtration was carried out by allowing the same buffer (pH 4.5) containing 1 M NaCl to flow through the column to measure the molecular weight. Pharmacia molecular weight markers 5 were used as standard proteins having known molecular weights to prepare a calibration curve. As a result, the molecular weight of the enzyme was about 150 kDa.

Based on the results of SDS-gel electrophoresis and gel filtration, the enzyme was suggested to be a homodimer having a 10 molecular weight of about 75 kDa.

#### Example 5 Enzyme Optimum pH

The effects of pH were examined in the reaction in which L-alanyl-L-glutamine is formed from L-alanine methyl ester 15 hydrochloride and L-glutamine. Acetate buffer (pH 3.9 to 5.4), MES buffer (pH 5.4 to 6.4), phosphate buffer (pH 6.0 to 7.9), borate buffer (pH 7.8 to 9.3), CAPS buffer (pH 9.3 to 10.7) and K<sub>2</sub>HPO<sub>4</sub>-NaOH buffer (pH 10.8 to 11.6) were used as buffers. 1 microliter (μl) of the Mono S fraction enzyme obtained in Example 3 (about 180 U/ml) was added to 20 100 μl of each buffer (100 mM containing 100 mM L-alanine methyl ester, 200 mM L-glutamine and 10 mM EDTA and allowed to react at 18°C for 5 minutes to measure the effects of pH on the reaction. The results based on assigning a value of 100% to the case of using borate buffer (pH 9.3) are shown in Fig. 1. As a result, the optimum enzyme 25 pH was 8 to 9.5.

### Example 6 Enzyme Optimum Temperature

The effects of temperature were examined on the reaction in which L-alanyl-L-glutamine is formed from L-alanine methyl ester

5 hydrochloride and L-glutamine. A 1  $\mu$ l aliquot of the same enzyme fraction used in Example 5 was added to 100  $\mu$ l of 100 mM borate buffer (pH 9.0) containing 100 mM L-alanine methyl ester, 200 mM L-glutamine and 10 mM EDTA and allowed to react for 5 minutes at each temperature to measure the effects of temperature on the reaction.

10 The results based on assigning a value of 100% to the activity at 34°C are shown in Fig. 2. As a result, the optimum enzyme temperature was 30 to 40°C.

### Example 7 Enzyme Inhibitors

15 The effects of inhibitors on the production of L-alanyl-L-glutamine were examined using L-alanine methyl ester hydrochloride and L-glutamine as substrates. A 2  $\mu$ l aliquot of the same enzyme fraction used in Example 5 was added to 50  $\mu$ l of 100 mM borate buffer (pH 9.0) containing each of the enzyme inhibitors shown

20 in Table 2 at 10 mM, and allowed to react at 25°C for 5 minutes. Note that, o-phenanthroline, phenylmethylsulfonyl fluoride and p-nitrophenyl-p'-guanidinobenzoate were dissolved in methanol to a concentration of 50 mM before use. The enzyme activity under each condition was indicated as the relative activity in the case of assigning

25 a value of 100 to the production of L-alanyl-L-glutamine in the absence

of enzyme inhibitor. Those results are shown in Table 2. As a result, among the serine enzyme inhibitors tested, the enzyme was not inhibited by phenylmethylsulfonyl fluoride, but it was inhibited by p-nitrophenyl-p'-guanidinobenzoate.

5

Table 2

Enzyme inhibitor		Relative activity of L-Ala-L-Gln production (%)
None		100
Metal enzyme inhibitor	EDTA	96
	o-Phenanthroline	96
SH enzyme inhibitor	N-Ethyl maleimide	110
	Monoiodoacetate	101
Serine enzyme inhibitor	Phenylmethylsulfonyl fluoride	115
	4-(2-Aminoethyl)benzene sulfonyl fluoride	75
	p-Nitrophenyl-p'-guanidino benzoate	0.1

Example 8 Production of L-Alanyl-L-Glutamine from L-Alanine Methyl Ester and L-Glutamine

A 3  $\mu$ l aliquot of the same enzyme fraction as used in Example 5 10 was added to 100  $\mu$ l of 100 mM borate buffer (pH 9.0) containing 100 mM L-alanine methyl ester hydrochloride, 200 mM L-glutamine and 10 mM EDTA, and allowed to react at 18°C. As a result, as shown in Fig. 3, 83 mM L-alanyl-L-glutamine (L-Ala-L-Gln) was formed in the case of an enzyme-added lot, and the concentration of by-product

L-Ala-L-Ala-L-Gln was 1.3 mM. On the other hand, there was hardly any production of L-Ala-L-Gln observed in an enzyme-non-added lot, and the enzyme concentration was only about 0.07 mM after reacting for 120 minutes.

5

**Example 9 Effects of L-Glutamine Concentration on Production of L-Alanyl-L-Glutamine**

A 1  $\mu$ l aliquot of the same enzyme fraction as used in Example 5 was added to 100  $\mu$ l of 100 mM borate buffer (pH 9.0) containing 100 mM L-alanine methyl ester hydrochloride, L-glutamine at the concentrations shown in Table 3 and 10 mM EDTA, and allowed to react at 18°C for 2 hours. Those results are shown in Table 3.

**Table 3**

L-Alanine methyl ester hydrochloride (mM)	L-Glutamine (mM)	L-Ala-L-Gln (mM)
100	100	68.2
	110	72.1
	120	73.3
	130	75.1
	150	75.5
	200	82.0

15

**Example 10 Enzyme Substrate Specificity (1)**

Ester specificity was examined in the case of using L-amino acid ester for the carboxy component. A 2  $\mu$ l aliquot of the same enzyme fraction as used in Example 5 was added to 100  $\mu$ l of 100 mM borate

buffer (pH 9.0) containing the carboxy components indicated in Table 4 at 100 mM, 200 mM L-glutamine and 10 mM EDTA, and allowed to react at 25°C for 2 hours. The amounts of L-Ala-L-Gln formed in this reaction are shown in Table 4 (HCl represents hydrochloride in Table 4).

5

Table 4

Carboxy component	L-Ala-L-Gln produced (mM)
L-Alanine methyl ester·HCl	84.3
L-Alanine ethyl ester·HCl	91.5
L-Alanine isopropyl ester·HCl	78.9
L-Alanine-t-butyl ester·HCl	7.5

Example 11 Enzyme Substrate Specificity (2)

Peptide production was examined in the case of using L-alanine methyl ester for the carboxy component and using various L-amino acids for the amine component. A 2 µl aliquot of the same enzyme fraction as used in Example 5 was added to 100 µl of 100 mM borate buffer (pH 9.0) containing 100 mM L-alanine methyl ester hydrochloride, the L-amino acids shown in Table 5 at 150 mM and 10 mM EDTA, and allowed to react at 25°C for 3 hours. The amounts of each of the peptides formed in this reaction are shown in Table 5. Note that the "+" mark indicates those peptides for which production was confirmed but which were unable to be quantified due to the absence of a standard, while "tr" indicates a trace amount.

Table 5

Amine com- ponent	Produced peptide (mM)		Amine com- ponent	Produced peptide (mM)	
Gly	L-Ala-Gly	13.7	L-Asn	L-Ala-L-Asn	65.5
L-Ala	L-Ala-L-Ala	25.4	L-Gln	L-Ala-L-Gln	79.3
L-Val	L-Ala-L-Val	20.8	L-Tyr	L-Ala-L-Tyr	17.6
L-Leu	L-Ala-L-Leu	45.3	L-CySH	L-Ala-L-CySH	+
L-Ile	L-Ala-L-Ile	33.9	L-Lys	L-Ala-L-Lys	71.8
L-Met	L-Ala-L-Met	83.3	L-Arg	L-Ala-L-Arg	88.0
L-Phe	L-Ala-L-Phe	74.4	L-His	L-Ala-L-His	66.9
L-Trp	L-Ala-L-Trp	53.9	L-Asp	L-Ala-L-Asp	2.1
L-Ser	L-Ala-L-Ser	62.5	L-Glu	L-Ala-L-Glu	42.9
L-Thr	L-Ala-L-Thr	53.9	L-Pro	L-Ala-L-Pro	tr

## Example 12 Enzyme Substrate Specificity (3)

Peptide production was examined in the case of using various types of L-amino acid methyl esters for the carboxy component and using L-glutamine for the amine component. A 2  $\mu$ l aliquot of the same enzyme fraction as used in Example 5 was added to 100  $\mu$ l of 100 mM borate buffer (pH 9.0) containing the L-amino acid methyl ester hydrochlorides (AA-OMe·HCl) shown in Table 6 at 100 mM, 150 mM L-glutamine and 10 mM EDTA, and allowed to react at 25°C for 3 hours. The amounts of each of the peptides formed in this reaction are shown in Table 6. Note that the "+" mark indicates those peptides for which production was confirmed but which were unable to be quantified due to the absence of a standard, while "tr" indicates a trace amount.

Furthermore, Tween-80 was added to the reaction system to a final concentration of 0.1% in the case of using L-Trp-OMe and L-Tyr-OMe.

Table 6

Carboxy component	Produced peptide (mM)		Carboxy component	Produced peptide (mM)	
Gly-OMe	Gly-L-Gln	54.7	L-Tyr-OMe	L-Tyr-L-Gln	3.4
L-Ala-OMe	L-Ala-L-Gln	74.6	CySH-OMe	L-CySH-L-Gln	+
L-Val-OMe	L-Val-L-Gln	15.4	L-Lys-OMe	L-Lys-L-Gln	+
L-Leu-OMe	L-Leu-L-Gln	+	L-Arg-OMe	L-Arg-L-Gln	7.1
L-Ile-OMe	L-Ile-L-Gln	8.4	L-His-OMe	L-His-L-Gln	+
L-Met-OMe	L-Met-L-Gln	12.0	L-Asp- $\alpha$ -OMe	$\alpha$ -L-Asp-L-Gln	tr
L-Phe-OMe	L-Phe-L-Gln	0.9	L-Asp- $\beta$ -OMe	$\beta$ -L-Asp-L-Gln	tr
L-Trp-OMe	L-Trp-L-Gln	+	L-Glu- $\alpha$ -OMe	$\alpha$ -L-Glu-L-Gln	+
L-Ser-OMe	L-Ser-L-Gln	24.0	L-Glu- $\gamma$ -OMe	$\gamma$ -L-Glu-L-Gln	+
L-Thr-OMe	L-Thr-L-Gln	81.9	L-Pro-OMe	L-Pro-L-Gln	2.2
L-Asn-OMe	L-Asn-L-Gln	+			
L-Gln-OMe	L-Gln-L-Gln	0.3			

5 Hydrochloride salts were used for all of the carboxy components.

#### Example 13 Enzyme Substrate Specificity (4)

Peptide production was examined in the case of using various L-amino acid methyl esters for the carboxy component and various L-amino acids for the amine component. A 2  $\mu$ l aliquot of the same enzyme fraction as used in Example 5 was added to 100  $\mu$ l of 100 mM borate buffer (pH 9.0) containing the L-amino acid methyl ester hydrochlorides (AA-OMe·HCl) shown in Table 7 at 100 mM, the L-amino

10

acids shown in Table 7 at 150 mM and 10 mM EDTA, and allowed to react at 25°C for 3 hours. The amounts formed of each of the peptides formed in this reaction are shown in Table 7. Note that the "tr" indicates a trace amount. Furthermore, Tween-80 was added to the 5 reaction system to a final concentration of 0.1% in the case of using L-Trp-OMe. Note that the "+" mark indicates those peptides for which production was confirmed but which were unable to be quantified due to the absence of a standard.

Table 7

Carboxy component	Amine component	Produced peptide (mM)		Carboxy component	Amine component	Produced peptide (mM)	
Gly-OMe	L-CySH	Gly-L-CySH	45.6	L-Met-OMe	L-Ser	L-Met-L-Ser	12.8
	L-Arg	Gly-L-Arg	25.5		L-Met	L-Met-L-Met	25.0
	L-Phe	Gly-L-Phe	44.0		L-Phe	L-Met-L-Phe	34.0
	L-His	Gly-L-His	31.6	L-Ile-OMe	L-Ser	L-Ile-L-Ser	17.2
	L-Lys	Gly-L-Lys	9.8		L-Met	L-Ile-L-Met	10.0
	L-Ser	Gly-L-Ser	44.2		L-Phe	L-Ile-L-Phe	5.2
L-Thr-OMe	Gly	L-Thr-Gly	9.4	L-Arg-OMe	L-Ser	L-Arg-L-Ser	3.6
	L-Ala	L-Thr-L-Ala	9.4		L-Met	L-Arg-L-Met	0.7
	L-Val	L-Thr-L-Val	0.7		L-Phe	L-Arg-L-Phe	1.9
	L-Leu	L-Thr-L-Leu	28.4	L-Leu-OMe	L-Met	L-Leu-L-Met	12.2
	L-Met	L-Thr-L-Met	38.6	L-Trp-OMe	L-Met	L-Trp-L-Met	4.1
	L-Ser	L-Thr-L-Ser	58.2	L-Lys-OMe	L-Met	L-Lys-L-Met	6.8
L-Ser-OMe	L-Ser	L-Ser-L-Ser	38.0	L-His-OMe	L-Met	L-His-L-Met	6.5
	L-Met	L-Ser-L-Met	12.5	L-Asn-OMe	L-Glu	LAsn-L-Glu	10.2
	L-Phe	L-Ser-L-Phe	20.3				
L-Val-OMe	L-Ser	L-Val-L-Ser	30.8				
	L-Met	L-Val-L-Met	10.3				
	L-Phe	L-Val-L-Phe	6.1				

Hydrochloride salts were used for all the carboxy components.

#### Example 14 Enzyme Substrate Specificity (5)

5 Peptide production was examined in the case of using the L or D forms of various amino acid methyl esters for the carboxy component, and the L or D forms of various amino acids for the amine component.

2  $\mu$ l of the same enzyme fraction as used in Example 5 was added to 100  $\mu$ l of 100 mM borate buffer (pH 9.0) containing the various amino acid methyl ester hydrochlorides (AA-OMe·HCl) shown in Table 8 at 100 mM, the various amino acids shown in Table 8 at 150 mM and 10 mM EDTA, and allowed to react at 25°C for 3 hours. The amounts of each of the peptides formed in this reaction are shown in Table 8. Note that the "tr" indicates a trace amount.

Table 8

Carboxy component	Amine component	Produced peptide	(mM)
D-Ala-OMe	L-Gln	D-Ala-L-Gln	69.3
D-Ala-OMe	L-Ser	D-Ala-L-Ser	20.3
D-Thr-OMe		D-Thr-L-Ser	1.0
D-Ser-OMe		D-Ser-L-Ser	3.3
D-Val-OMe		D-Val-L-Ser	0.6
D-Met-OMe		D-Met-L-Ser	5.1
L-Ala-OMe	D-Gln	L-Ala-D-Gln	0.3
L-Ala-OMe	D-Ser	L-Ala-D-Ser	5.4
L-Thr-OMe		L-Thr-D-Ser	6.9
L-Ser-OMe		L-Ser-D-Ser	16.2
L-Val-OMe		L-Val-D-Ser	1.4
L-Met-OMe		L-Met-D-Ser	1.9
D-Ala-OMe	D-Gln	D-Ala-D-Gln	tr
D-Ala-OMe	D-Ser	D-Ala-D-Ser	0.2
D-Thr-OMe		D-Thr-D-Ser	1.1
D-Ser-OMe		D-Ser-D-Ser	2.5
D-Val-OMe		D-Val-D-Ser	0.5
D-Met-OMe		D-Met-D-Ser	2.7

Hydrochloride salts were used for all the carboxy components.

#### Example 15 Enzyme Substrate Specificity (6)

5 Peptide production was examined using various L-amino acid amides for the carboxy component, and various L-amino acids for the amine component. A 2  $\mu$ l aliquot of the same enzyme fraction as that used in Example 5 was added to 100  $\mu$ l of 100 mM borate buffer (pH 9.0) containing the L-amino acid amide hydrochloride salts

10 (AA-NH<sub>2</sub>·HCl) shown in Table 9 at 100 mM, the L-amino acids shown in

Table 9 at 150 mM and 10 mM EDTA, and allowed to react at 25°C for 3 hours. The amounts of each of the peptides formed in this reaction are shown in Table 9.

Table 9

Carboxy component	Amine component	Produced peptide	(mM)
L-Phe-NH <sub>2</sub>	L-Gln	L-Phe-L-Gln	0.2
L-Phe-NH <sub>2</sub>	L-Ser	L-Phe-L-Ser	0.6
L-Ala-NH <sub>2</sub>	L-Gln	L-Ala-L-Gln	7.6
L-Ala-NH <sub>2</sub>	L-Met	L-Ala-L-Met	3.4
L-Ala-NH <sub>2</sub>	L-His	L-Ala-L-His	3.9
L-Thr-NH <sub>2</sub>	L-Gln	L-Thr-L-Gln	0.3

5

#### Example 16 Enzyme Substrate Specificity (7)

Peptide production was examined in the case of using various L-alanine methyl esters for the carboxy component and C-protected L-amino acids for the amine component. A 2 µl aliquot of the same 10 enzyme fraction as used in Example 5 was added to 100 µl of 100 mM borate buffer (pH 9.0) containing the L-alanine methyl ester hydrochloride (Ala-OMe·HCl) shown in Table 10 at 100 mM, the L-amino acid amide hydrochloride salts shown in Table 10 at 150 mM and 10 mM EDTA, and allowed to react at 25°C for 3 hours. The amounts of each 15 of the peptides formed in this reaction are shown in Table 10.

Table 10

Carboxy component	Amine component	Produced peptide	(mM)
L-Ala-OMe	Gly-NH <sub>2</sub>	L-Ala-Gly-NH <sub>2</sub>	7.4
	L-Ala-NH <sub>2</sub>	L-Ala-L-Ala-NH <sub>2</sub>	8.3
	L-Phe-NH <sub>2</sub>	L-Ala-L-Phe-NH <sub>2</sub>	12.2

Example 17 Enzyme Substrate Specificity (8)

Peptide production was examined in the case of using various 5 amino acid methyl esters for the carboxy component and methylamine for the amine component. A 2  $\mu$ l aliquot of the same enzyme fraction as used in Example 5 was added to 100  $\mu$ l of 100 mM borate buffer (pH 9.0) containing the amino acid methyl ester hydrochloride salt (AA-OMe·HCl) shown in Table 11 at 100 mM, the methylamine shown in 10 Table 11 at 150 mM and 10 mM EDTA, and allowed to react at 25°C for 3 hours. The amounts of each of the peptides formed in this reaction are shown in Table 11.

Table 11

Carboxy component	Amine component	Produced peptide	(mM)
Gly-OMe	Methylamine	Gly-methylamine	1.1
L-Thr-OMe		L-Thr-methylamine	0.2
L-Ala-OMe		L-Ala-methylamine	0.3

15 Example 18 Enzyme Substrate Specificity (9)

Peptide production was examined in the case of using  $\beta$ -amino

acid ester for the carboxy component or  $\beta$ -amino acid for the amine component. A 2  $\mu$ l aliquot of the same enzyme fraction as used in Example 5 was added to 100  $\mu$ l of 100 mM borate buffer (pH 9.0) containing the carboxy components shown in Table 12 at 100 mM, the 5 amine components shown in Table 12 at 150 mM and 10 mM EDTA, and allowed to react at 25°C for 3 hours. The amounts of each of the peptides formed in this reaction are shown in Table 12. Note that the "tr" indicates a trace amount.

Table 12

Carboxy component	Amine component	Produced peptide	(mM)
Gly-OMe	$\beta$ -Ala	Gly- $\beta$ -Ala	2.2
Gly-OMe	$\beta$ -Phe	Gly- $\beta$ -Phe	0.4
L-Ala-OMe	$\beta$ -Ala	Ala- $\beta$ -Ala	7.7
L-Ala-OMe	$\beta$ -Phe	Ala- $\beta$ -Phe	1.4
L-Thr-OMe	$\beta$ -Ala	Thr- $\beta$ -Ala	3.2
L-Thr-OMe	$\beta$ -Phe	Thr- $\beta$ -Phe	1.4
$\beta$ -Ala-OMe	L- $\alpha$ -Ala	$\beta$ -Ala-L- $\alpha$ -Ala	tr
$\beta$ -Ala-OMe	$\beta$ -Ala	$\beta$ -Ala- $\beta$ -Ala	0.2
B-Ala-OMe	L-Gln	$\beta$ -Ala-L-Gln	0.6
B-Ala-OMe	L-Ser	$\beta$ -Ala-L-Ser	3.2

10 Hydrochloride salts were used for all the carboxy components.

#### Example 19 Enzyme Substrate Specificity (10)

Oligopeptide production was examined in the case of using L-amino acid ester for the carboxy component and peptide for the

amine component. A 2  $\mu$ l aliquot of the same enzyme fraction as used in Example 5 was added to 100  $\mu$ l of 100 mM borate buffer (pH 9.0) containing the carboxy components shown in Table 13 at 100 mM, the amine components shown in Table 13 at 150 mM and 10 mM EDTA, and 5 allowed to react at 25°C for 3 hours. The amounts of each of the peptides formed in this reaction are shown in Table 13. As a result, it was clearly demonstrated that the present enzyme can form not only dipeptide, but also long-chain peptides by using a peptide for the amine component.

10

As has been indicated in the aforementioned Examples 9 to 19, the present enzyme obtained from *Empedobacter brevis* strain FERM P BP-8113 (Depository institution: the independent administrative corporation, National Institute for Advanced Industrial Science and 15 Technology, International Patent Organism Depository, Address of depository institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date: July 8, 2002) was determined to have an extremely broad substrate specificity.

Table 13

Carboxy component	Amine component	Produced peptide	(mM)
L-Ala-OMe	L-Ala	L-Ala-L-Ala	28.7
	L-Ala-L-Ala	L-Ala-L-Ala-L-Ala	57.5
	L-Ala-L-Ala-L-Ala	L-Ala-L-Ala-L-Ala-L-Ala	44.5
	L-Ala-L-Ala-L-Ala-L-Ala	L-Ala-L-Ala-L-Ala-L-Ala-L-Ala	34.8
	L-Ala-L-Ala-L-Ala-L-Ala-L-Ala	L-Ala-L-Ala-L-Ala-L-Ala-L-Ala-L-Ala	1.4*
	L-Ala-L-Gln	L-Ala-L-Ala-L-Gln	15.2
	Gly-L-Ala	L-Ala-Gly-L-Ala	25.9
	Gly-Gly	L-Ala-Gly-Gly	41.7
	L-His-L-Ala	L-Ala-L-His-L-Ala	55.9
	L-Leu-L-Ala	L-Ala-L-Leu-L-Ala	48.3
	L-Phe-L-Ala	L-Ala-L-Phe-L-Ala	49.7
	L-Phe-Gly	L-Ala-L-Phe-Gly	43.7
Gly-OMe	L-Ala-L-Tyr	Gly-L-Ala-L-Tyr	1.7
	Gly-L-Gln	Gly-Gly-L-Gln	7.2
	Gly-L-Tyr-L-Ala	Gly-Gly-L-Tyr-L-Ala	44.2
L-Thr-OMe	Gly-Gly	L-Thr-Gly-Gly	83.0

\*: Since the solubility of L-Ala-L-Ala-L-Ala-L-Ala-L-Ala was low, the carboxy component was used at a concentration of 10 mM and the amine component at 15 mM in this reaction system. The other 5 conditions were the same as explained in the example. Hydrochloride salts were used for all the carboxy components.

#### Example 20 Comparison of Ability to Catalyze Peptide Formation with

## Known Enzymes

The peptide formation ability of the present enzyme was compared with that of known enzymes. Carboxypeptidase Y described in EP 278787A1 and the thiol endopeptidases (ficin, papain, bromelain and chymopapain) described in EP 359399B1 were used for the known enzymes, and they were used in the form of purified enzymes manufactured by Sigma. The enzyme homogeneously purified in Example 3 was used for the source of the enzyme of the present invention. These enzymes were added to a reaction system in the amounts shown in Table 14 as protein. The reaction was carried out by adding enzyme to 100 µl of borate buffer (pH 9.0) containing 100 mM L-alanine methyl ester hydrochloride salt and 200 mM L-glutamine and allowing to react at 25°C. Furthermore, enzyme dissolved in 10 mM acetate buffer (pH 5.0) containing 1 mM EDTA was used for the carboxypeptidase, while enzyme dissolved in 10 mM acetate buffer (pH 5.0) containing 2 mM EDTA, 0.1 M KCl and 5 mM dithiothreitol was used for the thiol endopeptidase. The ratios of the formation rates of L-alanyl-L-glutamine by these enzymes are shown in Table 14.

As a result, the production of an extremely trace amount of L-alanyl-L-glutamine was observed even in the absence of enzyme, while a slight increase in the formation rate was observed in the lot where carboxypeptidase or thiol endopeptidase was added as compared with the lot where no enzyme was added. In contrast, an overwhelmingly faster rate of production of L-alanyl-L-glutamine was observed in the lot where the present enzyme was added, and that rate

of production was about 5,000 to 100,000 times faster than carboxypeptidase Y and thiol endopeptidase. As has been described above, the present enzyme was verified to have an extremely fast peptide formation rate unlike any enzyme in the prior art. Furthermore, 5 in contrast to the enzyme of the present invention being a dimer having a molecular weight of about 75,000, since the molecular weight of carboxypeptidase Y has been reported to be about 61,000, while that of thiol endopeptidase has been reported to be about 23,000 to 36,000, the L-alanyl-L-glutamine formation rate per molecular weight is even 10 greater for the enzyme of the present invention than that per unit weight indicated in the examples.

Table 14

Enzyme	Amount of enzyme added (protein mg/ml)	L-Ala-L-Gln production rate (mM/min)	Ratio of L-Ala-L-Gln production rate per enzyme unit weight
No enzyme	0	0.0006	
Carboxypeptidase Y	0.61	0.0257	0.0191
Ficin	2.60	0.0096	0.0017
Papain	2.30	0.0106	0.0021
Bromelain	2.80	0.0062	0.0010
Chymopapain	3.60	0.0100	0.0013
Enzyme of present invention	0.02	4.4000	100.0

Example 21 Production of L-Alanyl-L-Glutamine Using

Microbial Cells of *Sphingobacterium* sp.

A 50 ml medium (pH 7.0) containing 5 g of glucose, 5 g of ammonium sulfate, 1 g of monopotassium phosphate, 3 g of dipotassium phosphate, 0.5 g of magnesium sulfate, 10 g of yeast extract and 10 g of peptone in 1 L was transferred to a 500 mL Sakaguchi flask and sterilized at 115°C for 15 minutes for culturing *Sphingobacterium* sp. strain FERM BP-8124 (Depositary institution: the independent administrative corporation, National Institute for Advanced Industrial Science and Technology, International Patent Organism

5 10 15 20 25

Depositary, Address of depositary institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit date: July 22, 2002). This medium was then inoculated with one loopful of *Sphingobacterium* sp. strain FERM BP-8124 (Depositary institution: the independent administrative corporation, National Institute for Advanced Industrial Science and Technology, International Patent Organism

Depositary, Address of depositary institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit date: July 22, 2002) cultured at 30°C for 24 hours in a slant agar medium (agar: 20 g/L, pH 7.0) containing 5 g of glucose, 10 g of yeast extract, 10 g of peptone and 5 g of NaCl in 1 L, followed by shake culturing at 30°C for 20 hours and 120 strokes/minute. A 1 ml aliquot of this culture broth was then added to the aforementioned medium (50 ml/500 mL Sakaguchi flask) and cultured at 30°C for 18 hours. Following completion of the culturing, the microbial cells were separated from the culture broth by centrifugation and suspended in 0.1 M borate buffer

(pH 9.0) containing 10 mM EDTA to 100 g/L as wet microbial cells. A 0.1 mL aliquot of 100 mM borate buffer (pH 9.0) containing 10 mM EDTA, 200 mM L-alanyl methyl ester hydrochloride and 400 mM L-glutamine was then added to 0.1 mL of this microbial cell suspension, 5 and after bringing to a final volume of 0.2 mL, was allowed to react at 25°C for 120 minutes. The amount of L-alanyl-L-glutamine formed at this time was 62 mM.

**Example 22 Purification of Enzyme from *Sphingobacterium* sp.**

10 The following procedure after centrifugal separation was carried out either on ice or at 4°C. *Sphingobacterium* sp. strain FERM BP-8124 (Depository institution: the independent administrative corporation, National Institute for Advanced Industrial Science and Technology, International Patent Organism Depository, Address of 15 depository institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit date: July 22, 2002) was cultured in the same manner as Example 21, and the microbial cells were collected by centrifugal separation (10,000 rpm, 15 minutes). After washing 2 g of microbial cells with 20 mM Tris-HCl buffer (pH 7.6), 20 they were suspended in 8 ml of the same buffer and subjected to ultrasonic crushing treatment for 45 minutes at 195 W. This ultrasonic crushing liquid was then centrifuged (10,000 rpm, 30 minutes) to remove the crushed cell fragments and obtain an ultrasonic crushing liquid supernatant. This ultrasonic crushing liquid supernatant was 25 dialyzed overnight against 20 mM Tris-HCl buffer (pH 7.6) followed by

removal of the insoluble fraction by ultracentrifugation (50,000 rpm, 30 minutes) to obtain a soluble fraction in the form of the supernatant liquid. The resulting soluble fraction was applied to a Q-Sepharose HP column (manufactured by Amersham) pre-equilibrated with Tris-HCl 5 buffer (pH 7.6), and the active fraction was collected from the non-adsorbed fraction. This active fraction was dialyzed overnight against 20 mM acetate buffer (pH 5.0) followed by removal of the insoluble fraction by centrifugal separation (10,000 rpm, 30 minutes) to obtain a dialyzed fraction in the form of the supernatant liquid. This 10 dialyzed fraction was then applied to an SP-Sepharose HP column (manufactured by Amersham) pre-equilibrated with 20 mM acetate buffer (pH 5.0) to obtain the active fraction in which enzyme was eluted at a linear concentration gradient of the same buffer containing 0 to 1 M NaCl.

15

#### Example 23 Production of L-Alanyl-L-Glutamine Using Enzyme Fraction

A 10  $\mu$ l aliquot of the SP-Sepharose HP fraction (about 27 U/ml) purified in Example 22 was added to 90  $\mu$ l of 111 mM borate buffer (pH 20 9.0) containing 111 mM L-alanine methyl ester hydrochloride, 222 mM L-glutamine and 11 mM EDTA, and allowed to react at 25°C for 120 minutes. As a result, 73 mM of L-alanyl-L-glutamine was formed in the enzyme-added lot. On the other hand, there was hardly any production of L-Ala-L-Glu observed in the enzyme-non-added lot, and 25 the production amount was only about 0.07 mM after reacting for 120

minutes.

#### Example 24 Enzyme Substrate Specificity (11)

Substrate specificity was examined for enzyme derived from

- 5 *Sphingobacterium sp.* strain FERM BP-8124 (Depository institution: the independent administrative corporation, National Institute for Advanced Industrial Science and Technology, International Patent Organism Depository, Address of depositary institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit date: 10 July 22, 2002). 100 µl of 100 mM borate buffer (pH 9.0) containing the various carboxy components at a final concentration of 100 mM and the various amine components at a final concentration of 150 mM shown in Tables 15-1 to 15-4, the SP-Sepharose HP fraction enzyme purified in Example 22 (addition of 0.33 units in the reaction liquid) and 10 mM 15 EDTA were allowed to react at 25°C for 1.5 hours. The amounts of each of the peptides formed in this reaction are shown in Table 15. Note that the "+" mark indicates those peptides for which production was confirmed but which were unable to be quantified due to the absence of a standard, while "tr" indicates a trace amount.
- 20 Furthermore, Tween-80 was added to the reaction system to a final concentration of 0.1% in the case of using L-Tyr-OMe. In addition, hydrochlorides were used for all carboxy components.

Table 15-1

Carboxy component	Amine component	Produced peptide (mM)
L-Ala-OMe	Gly	L-Ala-Gly 11.1
	L-Ala	L-Ala-L-Ala 13.1
	L-Val	L-Ala-L-Val 10.9
	L-Leu	L-Ala-L-Leu 33.0
	L-Ile	L-Ala-L-Ile 24.7
	L-Met	L-Ala-L-Met 86.9
	L-Pro	L-Ala-L-Pro 1.5
	L-Phe	L-Ala-L-Phe 69.5
	L-Trp	L-Ala-L-Trp 46.0
	L-Thr	L-Ala-L-Thr 47.3
	L-Asn	L-Ala-L-Asn 52.3
	L-Tyr	L-Ala-L-Tyr 11.1
	L-CySH	L-Ala-L-CySH +
	L-Lys	L-Ala-L-Lys 71.2
	L-Arg	L-Ala-L-Arg 72.2
	L-His	L-Ala-L-His 73.6
	L-Asp	L-Ala-L-Asp 2.3
	L-Glu	L-Ala-L-Glu 39.1
D-Ala-OMe	L-Ser	L-Ala-L-Ser 43.8
	D-Ser	L-Ala-D-Ser 3.3
	L-Ser	D-Ala-L-Ser 24.1
	D-Ser	D-Ala-D-Ser 5.5

Table 15-2

Carboxy component	Amine component	Produced peptide (mM)
L-Thr-OMe	L-Gln	L-Thr-L-Gln 36.1
Gly-OMe		Gly-L-Gln 61.1
L-Ser-OMe		L-Ser-L-Gln 12.9
L-Val-OMe		L-Val-L-Gln 8.2
L-Met-OMe		L-Met-L-Gln 32.6
L-Ile-OMe		L-Ile-L-Gln 6.4
L-Arg-OMe		L-Arg-L-Gln 17.2
L-Tyr-OMe		L-Tyr-L-Gln 0.6
L-Pro-OMe		L-Pro-L-Gln 1.8
L-Phe-OMe		L-Phe-L-Gln 0.8
L-Gln-OMe		L-Gln-L-Gln 0.1
Asp- $\alpha$ -OMe		$\alpha$ -L-Asp-L-Gln 0.05

Table 15-3

Carboxy component	Amine component	Produced peptide (mM)	
L-Thr-OMe	Gly	L-Thr-Gly	0.4
	L-Ala	L-Thr-L-Ala	5.8
	L-Val	L-Thr-L-Val	1.3
	L-Leu	L-Thr-L-Leu	15.3
	L-Met	L-Thr-L-Met	28.9
Gly-OMe	L-Arg	Gly-L-Arg	17.9
	L-Phe	Gly-L-Phe	20.0
	L-His	Gly-L-His	36.2
	L-Lys	Gly-L-Lys	48.2
	L-Ser	Gly-L-Ser	53.8
L-Ser-OMe	L-Ser	L-Ser-L-Ser	9.9
	L-Met	L-Ser-L-Met	7.6
	L-Phe	L-Ser-L-Phe	4.3
L-Val-OMe	L-Ser	L-Val-L-Ser	31.9
	L-Met	L-Val-L-Met	6.8
	L-Phe	L-Val-L-Phe	1.0
L-Met-OMe	L-Ser	L-Met-L-Ser	25.3
	L-Met	L-Met-L-Met	28.4
	L-Phe	L-Met-L-Phe	8.9
L-Ile-OMe	L-Ser	L-Ile-L-Ser	17.3
	L-Met	L-Ile-L-Met	5.1
	L-Phe	L-Ile-L-Phe	1.5
L-Arg-OMe	L-Ser	L-Arg-L-Ser	2.2
	L-Met	L-Arg-L-Met	tr
	L-Phe	L-Arg-L-Phe	tr

Table 15-4

Carboxy component	Amine component	Produced peptide (mM)	
L-Ala-OMe	Gly amide	L-Ala-Gly amide	15.1
	L-Ala amide	L-Ala-L-Ala amide	9.2
	L-Phe amide	L-Ala-Phe amide	27.1
L-Ala-OMe	Methylamine	L-Ala-methylamine	0.6
L-Thr-OMe		L-Thr-methylamine	0.3
Gly-OMe		Gly-methylamine	1.0
L-Ala amide	L-Gln	L-Ala-L-Gln	0.3
	L-Met	L-Ala-L-Met	tr
	L-His	L-Ala-L-His	tr

Hydrochloride salts were used for all the carboxy components.

#### Example 25 Enzyme Substrate Specificity (12)

5 Substrate specificity with respect to oligopeptide production was examined for enzyme derived from *Sphingobacterium* sp. strain FERM BP-8124 (Depository institution: the independent administrative corporation, National Institute for Advanced Industrial Science and Technology, International Patent Organism Depository, Address of 10 depositary institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit date: July 22, 2002). A 100  $\mu$ l aliquot of 100 mM borate buffer (pH 9.0) containing the various carboxy components at a final concentration of 100 mM and the various amine components at a final concentration of 150 mM shown in Table 16, the

SP-Sepharose HP fraction enzyme purified in Example 22 (addition of 0.33 units in the reaction liquid) and 10 mM EDTA were allowed to react for 1.5 hours at 25°C. The amounts of each oligopeptide formed in this reaction are shown in Table 16. Furthermore, hydrochloride salts were 5 used for all carboxy components.

Table 16

Carboxy component	Amine component	Produced peptide (mM)	
L-Ala-OMe	L-Ala	L-Ala-L-Ala	25.6
	L-Ala-L-Ala	L-Ala-L-Ala-L-Ala	41.1
	L-Ala-L-Ala-L-Ala	L-Ala-L-Ala-L-Ala-L-Ala	30.1
	L-Ala-L-Ala-L-Ala-L-Ala	L-Ala-L-Ala-L-Ala-L-Ala-L-Ala	22.8
	Gly-Gly	L-Ala-Gly-Gly	33.7
	Gly-Ala	L-Ala-Gly-L-Ala	35.1
	L-His-L-Ala	L-Ala-L-His-L-Ala	58.0
	L-Phe-Gly	L-Ala-L-Phe-Gly	34.0
	L-Leu-L-Ala	L-Ala-L-Leu-L-Ala	40.7
	L-Phe-L-Ala	L-Ala-L-Phe-L-Ala	24.8
L-Thr-OMe	Gly-Gly	L-Thr-Gly-Gly	8.4
Gly-OMe	L-Ala-L-Tyr	Gly-L-Ala-L-Tyr	0.6

Example 26 Enzyme Substrate Specificity (13)

Substrate specificity was additionally assessed using the same 10 enzyme fraction as that used in Example 5.

Table 17

Carboxy component (mM)	Amine component (mM)	Produced peptide (mM)	Reaction time (hr)
H-Ala-OMe 50mM	H-p-F-Phe-OH 50mM	H-Ala-p-F-Phe-OH 21.9mM	3
H-Ala-OMe 40mM	H-Cl-F-Phe-OH 40mM	H-Ala-Cl-F-Phe-OH 20.8mM	3
H-Ala-OMe 40mM	H-p-NO <sub>2</sub> -Phe-OH 40mM	H-Ala-p-NO <sub>2</sub> -Phe-OH 27.5mM	3
H-Ala-OMe 100mM	H-t-Leu-OH 150mM	H-Ala-t-Leu-OH 0.4mM	3
H-Ala-OMe 20mM	H-2-Nal-OH 20mM	H-Ala-2-Nal-OH +	3
H-p-F-Phe-OMe 100mM	H-Gln-OH 150mM	H-p-F-Phe-H-Gln-OH tr	3
H-Cl-F-Phe-OMe 25mM	H-Gln-OH 50mM	H-Cl-F-Phe-H-Gln-OH tr	3
H-p-NO <sub>2</sub> -Phe-OMe 40mM	H-Gln-OH 40mM	H-p-NO <sub>2</sub> -Phe-H-Gln-OH 1.1mM	3
H-t-Leu-OMe 100mM	H-Gln-OH 150mM	H-t-Leu-H-Gln-OH tr	3
H-2-Nal-OMe 40mM	H-Gln-OH 40mM	H-2-Nal-H-Gln-OH tr	3
H-Aib-OMe 100mM	H-Gln-OH 150mM	H-Aib-H-Gln-OH 18.8mM	3
H-N-Me-Ala-OMe 100mM	H-Gln-OH 150mM	H-N-Me-Ala-H-Gln-OH 0.5mM	3
H-Aib-OMe 100mM	H-Phe-OH 150mM	H-Aib-Phe-OH 17.2mM	3
H-CHA-OMe 40mM	H-Phe-OH 40mM	H-CHA-Phe-OH +	3
H-N-Me-Ala-OMe 100mM	H-Phe-OH 150mM	H-N-Me-Ala-Phe-OH tr	3
H-Ala-OMe 100mM	H-Ser(tBu)-OH 150mM	H-Ala-Ser(tBu)-OH 48.8mM	2
H-Ser(tBu)-OMe 100mM	H-Gln-OH 150mM	H-Ser(tBu)-Gln-OH tr	2
H-Ala-OMe 100mM	H-Asp(OtBu)-OH 150mM	H-Ala-Asp(OtBu)-OH 62.6mM	2
H-Asp(OtBu)-OMe 100mM	H-Gln-OH 150mM	H-Asp(OtBu)-Gln-OH 0.9mM	2
H-Ala-OMe 100mM	H-Lys(Boc)-OH 150mM	H-Ala-Lys(Boc)-OH 51.0mM	2
H-Lys(Boc)-OMe 100mM	H-Gln-OH 150mM	H-Lys(Boc)-Gln-OH +	2

100  $\mu$ l of reaction solutions consisting of 100 mM borate buffer (pH 9.0) containing each of the carboxy components and amine  
5 components at the final concentrations shown in Table 17, enzyme

(addition of 0.1 unit in reaction solution) and 10 mM EDTA were allowed to react at 25°C for the reaction times shown in Table 17. The amounts of each of the peptides formed in the reactions are shown in Table 17. Note that a "+" mark indicates those for which production 5 was confirmed but which were unable to be quantified due to the absence of a standard, while "tr" indicates a trace amount.

Abbreviations:

H-Ala-OMe: L-alanine methyl ester hydrochloride

10 H-p-F-Phe-OMe: p-fluoro-L-phenylalanine methyl ester hydrochloride

H-Cl-F-Phe-OMe: p-chloro-L-phenylalanine methyl ester hydrochloride

H-p-NO<sub>2</sub>-Phe-OMe: p-nitro-L-phenylalanine methyl ester hydrochloride

H-t-Leu-OMe: tert-L-leucine methyl ester hydrochloride

H-2-Nal-OMe: 3-(2-naphthyl)-L-alanine methyl ester hydrochloride

15 H-Aib-OMe:  $\alpha$ -aminoisobutyric acid methyl ester hydrochloride

H-N-Me-Ala-OMe: N-methyl-L-alanine methyl ester hydrochloride

H-CHA-OMe:  $\beta$ -cyclohexyl-L-alanine methyl ester hydrochloride

H-Ser(tBu)-OMe: O-tert-butyl-L-serine methyl ester hydrochloride

H-Asp(OtBu)-OMe: L-aspartic acid  $\beta$ -tert-butyl ester  $\alpha$ -methyl ester

20 hydrochloride

H-Lys(Boc)-OMe: N- $\epsilon$ -tert-butoxycarbonyl-L-lysine methyl ester hydrochloride

H-p-F-Phe-OH: p-fluoro-L-phenylalanine

H-Cl-F-Phe-OH: p-chloro-L-phenylalanine

25 H-p-NO<sub>2</sub>-Phe-OH: p-nitro-L-phenylalanine

H-t-Leu-OH: tert-L-leucine  
H-2-Nal-OH: 3-(2-naphthyl)-L-alanine  
H-Gln-OH: L-glutamine  
H-Phe-OH: L-phenylalanine  
5 H-Ser(tBu)-OH: O-tert-butyl-L-serine  
H-Asp(OtBu)-OH: L-aspartic acid  $\beta$ -tert-butyl ester  
H-Lys(Boc)-OH: N- $\epsilon$ -tert-butoxycarbonyl-L-lysine

Example 27 Enzyme Substrate Specificity (14)

10 Substrate specificity with respect to oligopeptide production was assessed using the same enzyme fraction as Example 26. 100  $\mu$ l of reaction solutions consisting of 100 mM borate buffer (pH 9.0) containing each of the carboxy components and amine components at the final concentrations shown in Table 18, enzyme (the numbers of 15 units added to the reaction solution are described in Table 18) and 10 mM EDTA were allowed to react at 25°C for 3 hours. The amounts of each of the oligopeptides formed in the reactions are shown in Table 18. Note that the "+" mark indicates those for which production was confirmed but which were unable to be quantified due to the absence of 20 a standard, while "tr" indicates a trace amount. It should be noted that hydrochlorides were used for all of the carboxy components.

Table 18

Carboxy component	Amine component	Amount of enzyme (units)	Produced peptide (mM)	
Gly-OMe	L-Phe-L-Met	1.0	Gly-Phe-Met	13.3
L-Ala-OMe	L-Phe-L-Met	0.2	L-Ala-L-Phe-L-Met	+
L-Tyr-OMe	Gly-Gly-L-Phe-L-Met	1.0	L-Tyr-Gly-Gly-L-Phe-L-Met	2.7
L-Ala-OMe	Gly-Gly-L-Phe-L-Met	0.2	L-Ala-Gly-Gly-L-Phe-L-Met	+
Gly-OMe	Gly-L-Phe	0.1	Gly-L-Phe	17.3
L-Ala-OMe	Gly-L-Phe	0.1	L-Ala-Gly-L-Phe	+
D-Ala-OMe	Gly-L-Phe	0.1	D-Ala-Gly-L-Phe	Tr

Although the invention has been described with respect to a specific embodiment for a complete and clear disclosure, the appended 5 claims are not to be thus limited but are to be construed as embodying all modifications and alternative constructions that may occur to one skilled in the art which fairly fall within the basic teaching herein set forth.